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Purinergic modulation of mesangial extracellular matrix production: Role in diabetic and other glomerular diseases

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Purinergic modulation of mesangial extracellular matrix production: Role in diabetic and other glomerular diseases.

Background. Extracellular adenosine triphosphate (ATP) (eATP) mediates several biologic activities via purinergic P2 receptors (P2Rs). This study aimed at (*1*) evaluating the role of the purinergic system in modulating mesangial extracellular matrix (ECM) and transforming growth factor-β (TGF-β) production and (*2*) its contribution to diabetes-induced mesangial ECM accumulation.

Methods. Rat mesangial cells were grown in normal glucose (5.5 mmol/L) or high glucose (30 mmol/L) containing media and probed with purinergic agonists and antagonists for the assessment of the expression pattern and function of P2Rs; release of ATP and activity of ectoATPases; and changes in ECM and TGF-β expression.

Results. Cells cultured in normal glucose and high glucose expressed similar amounts of functional P2Rs of the $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ subtypes. Levels of eATP were higher in high glucose vs. normal glucose, with unchanged ectoATPase activity. The ATP-hydrolyzing enzymes hexokinase or apyrase reduced ECM and TGF-β production from cells grown in high glucose, but not normal glucose. Under both normal glucose and high glucose conditions, ATP and the $P2X_7$ agonist benzoylbenzoylATP increased dose-dependently ECM and TGF-β production, whereas the P2Y agonist uridine triphosphate produced the opposite effect. The $P2X_7$ inhibitor oxidized ATP attenuated the ECM and TGF-β up-regulation induced by ATP and, to a lesser extent, that caused by high glucose. A TGF-β neutralizing antibody also prevented ATP-induced ECM up-regulation.

Conclusion. These data indicate a role for eATP in regulating ECM production via TGF-β and suggest that P2XRs and P2YRs differentially modulate this process. An increased ATP release induced by hyperglycemia might contribute to mesangial matrix expansion occurring in diabetes.

Key words: extracellular ATP, purinergic receptors, mesangial cell, extracellular matrix, diabetic nephropathy.

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Purinergic nucleotides and receptors represent an autocrine/paracrine system that participates in the regulation of cell function. Release of adenosine triphosphate (ATP) from the cell can occur by conductive transport, facilitated transport, and exocytosis. In the extracellular space, ATP is degraded by ectoenzymes such as ectonucleotide triphosphate diphosphohydrolases [that degrade both ATP and adenosine diphosphate (ADP) to adenosine monophosphate (AMP) and inorganic phosphate], ectonucleotide pyrophosphatases/phosphodiesterases (that degrade ATP to AMP and pyrophosphate) or alkaline phosphatase. AMP is then hydrolyzed by 5′-nucleotidase to generate adenosine [1]. ATP and ADP bind to G protein–coupled purinergic P2Y receptors (P2YRs), with consequent activation of phospholipase C-β, generation of diacylglycerol and inositol-3-phosphate, activation of protein kinase C (PKC), and release of calcium from intracellular stores [2, 3]. ATP also binds to and activates purinergic P2X receptors (P2XRs), thus causing transmembrane calcium, sodium and potassium fluxes, with consequent membrane depolarization that may drive further calcium influx [2, 3]. AMP and adenosine interact with purinergic P1 receptors, with effects depending on the receptor subtype(s) [4]. While it was long believed that extracellular ATP (eATP) levels were basically negligible, it is now clear that presence of eATP in the pericellular space is the rule rather than the exception in several physiologic and pathologic conditions [1], thus leading to differential modulation of P2R activity even in the absence of added ATP [5].

It has been shown that P2YRs and P2XRs are expressed along the nephron and renal vasculature [1, 6] and that ATP is released by renal tubular epithelial cells [7], endothelial cells [8], smooth muscle cells [8], platelets [8], and nerves (as a cotransmitter of norepinephrine) [9], thus suggesting that the purinergic system exerts several physiologic functions at the kidney level by acting in

an autocrine/paracrine manner. The data available so far show that this system regulates renal microvascular function, glomerular capillary pressure and tubuloglomerular feedback [10, 11]. It is also involved in the control of glomerular volume through at least two mechanisms: an ATP-dependent glomerular relaxation, mediated by P2YRs possibly at the endothelial level, and a subsequent contraction, caused by either ATP itself (acting on mesangial cells or podocytes) or adenosine formed by ATP hydrolysis [12]. $P2X_7$ as well as $P2Y_2$, $P2Y_4$ and $P2Y_6$ were found to be expressed in rat mesangial cells [13, 14] and to exert opposite effects on cell turnover. The P2YRs (likely $P2Y_2$ and $P2Y_4$) were shown to promote mesangial cell proliferation [13, 15], via activation of mitogen-activated protein kinase (MAPK) [16] and induction of immediate early genes [17]. Conversely, $P2X_7$ was shown to induce mesangial cell death by apoptosis and necrosis [13, 18].

Together with altered glomerular cell turnover, abnormal extracellular matrix (ECM) deposition, resulting from an imbalance between synthesis and degradation, represents the main process underlying virtually all forms of renal disease, including diabetic nephropathy [19]. Mesangial cells make a major contribution to this sclerosing process, by producing increased amounts of matrix proteins in response to hyperglycemia [20], and transforming growth factor-β (TGF-β) is considered the main cytokine modulating ECM accumulation, since it promotes matrix synthesis and reduces its degradation [21].

This study using rat mesangial cells grown under normal glucose and high glucose conditions was aimed at evaluating (*1*) the role of the purinergic system in modulating mesangial ECM and TGF-β production and (*2*) its contribution to the accumulation of matrix proteins within the mesangium in diabetes.

METHODS

Design

Rat mesangial cells were cultured in media containing normal glucose (5.5 mmol/L) or high glucose (30 mmol/L) concentrations and probed with various purinergic agonists and antagonists. These agents included (*1*) the enzymes degrading endogenous ATP, hexokinase (50 μg/mL), and apyrase (4 U/mL), added for 4 hours; (*2*) the P2R agonists ATP, benzoylbenzoylATP (Bz-ATP), uridine triphosphate (UTP), and 2-methylthioadenosine-5′-triphosphate (2-MeS-ATP) at increasing doses (0.001, 0.01, 0.05, 0.15, 0.30, 0.50, and 1 mmol/L) for 4 hours; (*3*) the P2R antagonists pyridoxalphosphate-6-azophenyl-2′,4′,-disulfonic acid (PPADS), oxidized ATP (oATP) and 2′-deoxy-N^6-methyladenosine 3′,5′-bisphosphate (MRS-2179), given for 2 hours at the dosage of 0.30 mmol/L either alone or followed by coincubation with equimolar ATP for further 4 hours; and (*4*) a TGF-β blocking antibody or control chicken IgG, added for 4 hours at the concentration of 30 μg/mL together with 0.30 mmol/L ATP. All these experiment had a control for osmolarity [i.e., media containing iso-osmolar mannitol concentrations (5.5 mmol/L glucose + 24.5 mmol/L mannitol)]. All reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), except for MRS-2179, obtained from Tocris Cookson Ltd. (Bristol, UK), and TGF-β blocking antibody and control chicken IgG, purchased from R&D Systems (Minneapolis, MN, USA).

Under these experimental conditions, the following parameters were assessed: (*1*) expression pattern and function of purinergic receptors; (*2*) release of ATP and activity of ecto-ATPases; and (*3*) changes in ECM and TGF-β mRNA and protein expression in response to purinergic agonists and antagonists.

Cell culture

Rat mesangial cells were isolated and characterized as previously described [22]. For the experimental procedures, rat mesangial cells between the third and the tenth passage were cultured for 10 to 15 days (over two to three passages) in Dulbecco's modified Eagle's medium (Sigma Chemical Co.) supplemented with 17% fetal bovine serum, 2 mmol/L L-glutamine, and antibiotics, at 37°C in 95% air-5% CO_2 humidified atmosphere [28], under the glucose concentrations indicated above.

Expression and functional activity of purinergic receptors

P2YR and P2XR mRNA levels were measured by reverse transcription-polymerase chain reaction (RT-PCR) from total RNA extracts obtained by the acid guanidinium thiocianate-phenol-chloroform method using TRIzol® Reagent (Invitrogen Italia SRL, San Giuliano Milanese, Italy). Total RNA was reverse transcribed and resulting cDNA was amplified using Access RT-PCR System (Promega, Madison, WI, USA). Amplification primers for P2YR and P2XR subtypes and the housekeeping gene β-actin were the following: $P2Y_1$ sense 5′-CAT CTC CCC CAT TCT CTT-3′ and antisense 5′-GTT GCT TCT TCT TGA CCT GT-3′; $P2Y_2$ sense 5′-ACC CGC ACC CTC TAT TAC T-3′ and antisense 5′-CTT AGA TAC GAT TCC CCA ACT-3′; $P2Y_4$ sense 5′-TGG GTG TTT GGT TGG TAG TA-3′ and antisense 5′-GTC CCC CGT GAA GAG ATA G-3′; $P2Y_6$ sense 5′-GTT ATG GAG CGG GAC AAT GG-3′ and antisense 5′-AGG ATG CTG CCG TGT AGG TT-3′; $P2X_1$ sense 5′-CAT TGT GCA GAG AAC CCA GAA-3′ and antisense 5′-ATG TCC TCC GCA TAC TTG AAC-3′; $P2X_2$ sense 5′-GTT CAC AGC TCT ACC TGC-3′ and antisense 5′-GAT GAC TCC AAT GAC ACC-3′; $P2X_3$ sense 5′-TTC TTC ACC TAC GAG ACT ACC-3′ and antisense 5′-TTA ACC ACA TCC CCT ACC-3′; $P2X_4$ sense 5′-AGG GCT ACC AGG AAA CGG AC-3′ and antisense 5′-GAT

TGT GCC AAG ACG GAA TA-3′; $P2X_5$ sense 5′-ATC TCT ACT GTC CCA TCT TCC-3′ and antisense 5′-TTG CTA TTC TGC TTC CTC C-3′; $P2X_6$ sense 5′-TAA CCA ACT TCC TTG TGA CAC C-3′ and antisense 5′-TGA AAT TGT ACC CCC TCT CC-3′; $P2X_7$ sense 5′-AGG AGC CCC TTA TCA GCT CT-3′ and antisense 5′-CAT TGG TGT ACT TGT CGT CC-3′; and β-actin sense 5′-TGG GAA TGG GTC AGA AGG ACT-3′ and antisense 5′-TTT CAC GGT TGG CCT TAG GGT-3′. Oligonucleotides were synthesized by M-Medical Genenco-Life Science (Florence, Italy). Amplification products were separated in 2% agarose gels, stained with ethidium bromide and quantified by scanning densitometry.

For $P2X_7$, protein expression was verified by Western blot analysis. Cells were lysed in lysis buffer [300 mmol/L sucrose, 1 mmol/L K_2HPO_4, 1 mmol/L $MgSO_4$, 5.5 mmol/L glucose, 20 mmol/L Hepes, pH 7.4, 1 mmol/L benzamidine, 1 mmol/L phenylmethylsulphonyl fluoride, 0.2 μg of DNase, and 0.2 μg of RNase] by freeze/thawing for three cycles. Lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5%) and electrophoretically transferred to nitrocellulose paper. Filters were then probed with a rabbit polyclonal anti-$P2X_7$ antibody raised against the synthetic peptide corresponding to the last 20 amino acids of $P2X_7$ protein (Alomone, Israel), used at a 1:100 dilution in Tris-buffered saline, followed by a goat antirabbit IgG antibody conjugated to alkaline phosphatase. Immunocomplexes were revealed by autoradiography and quantified by scanning densitometry. Results were normalized to the signal of connexin 43, as revealed by the use of a rabbit polyclonal antibody (Sigma Chemical Co.) at 1:5000 dilution. The expression of this cytoskeleton and gap junction protein was preliminary shown to be unaffected by high glucose.

Functional activity of these receptors was confirmed by assessing intracellular calcium mobilization in response to 1 mmol/L ATP. Changes in intracellular calcium concentration ($[Ca^{2+}]_i$) were measured in rat mesangial cells monolayers using Fura-2/AM, as previously reported [23]. Experiments were performed in a Perkin-Elmer LS50 fluorimeter (Perkin-Elmer, Beaconsfield, UK) equipped with a thermostat-controlled (37°C) cuvette holder and magnetic stirring. In addition, functional activity of $P2X_7$ despite low level expression was verified by immunofluorescence analysis of pore formation by this receptor [24]. Briefly, cells seeded on glass coverslips were rinsed with phosphate-buffered saline (PBS) and fixed with 2% paraformaldehyde in PBS. Then, fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 30 minutes, incubated with 2% fetal calf serum in PBS for 20 minutes, rinsed, and incubated overnight at 4°C with the rabbit anti-$P2X_7$ polyclonal antibody at 1:50 dilution. The next day, cells were incubated for 30 minutes with a fluorescein isothyocianate-conjugated goat antirabbit IgG, diluted 1:200, rinsed, and analyzed with a TE-300 Nikon (Nikon Co., Tokyo, Japan) fluorescence microscope.

Levels of ATP and activity of ecto-ATPases

ATP levels were measured by luminometric assay [25], using the ATP-Lite Luminescence ATP Detection Assay System (Perkin-Elmer, Boston, MA, USA), that has the advantages over other systems of higher sensitivity (down to five cells in 100 μL medium) and reproducibility, longer half-life of the light emission (>5 hours) and, particularly, more efficient inactivation of ectoenzymes (by raising the pH of the cell culture medium through the addition of the mammalian cell lysis solution), thus avoiding underestimation of eATP concentration. Briefly, rat mesangial cells were seeded at a concentration of 5×10^4 cells/well in microtiter plastic dishes in a total volume of culture medium of 100 μL, rinsed and supplemented with the substrate solution (luciferase + D-luciferin). Then, cells were placed directly into the test chamber of a luminometer (Victor 3) (Perkin-Elmer) and light emission was recorded in order to obtain eATP levels. Finally, cells were treated with a lysis solution, shaken for 5 minutes, and measurement was repeated to determine total ATP levels; intracellular ATP was calculated by subtraction.

The activity of ecto-ATPases was measured spectrophotometrically in the presence of 1 mmol/L ATP, as described by Ames [26]. Briefly, $\sim 3 \times 10^5$ cells were placed in 24-well plates, washed with phosphate-free saline solution, and incubated with 1 mmol/L ATP at 37°C for 15 minutes (for these cells, the ecto-ATPase activity is maximum after this time of incubation). The medium was then collected, centrifuged at $1100 \times g$ for 10 min and 300 μL of supernatant were challenged with 700 μL of reagent (one part of 10% ascorbic acid and six parts of 0.42% ammonium molybdate in 1 N K_2SO_4) at 37°C for 1 hour. Finally, phosphate released by this reaction was quantified by recording absorbance at 820 nm. Sensitivity of this method was 0.1 to 0.5 μmol/L inorganic phosphate.

ECM and TGF-β production

Transcripts for the ECM components fibronectin, laminin B1, and collagen IV α1 chain and the prosclerotic cytokine TGF-β1 were assessed by competitive RT-PCR [27]. Briefly, 1 μg of total RNA, extracted as previously described, was reverse transcribed using Retroscript Kit (Ambion, Austin, TX, USA). The following primers were used: fibronectin sense 5′-AGC GGT GTG GTC TAC TCT GT-3′ and antisense 5′-GAT GCA CTG ATC TCG GAF CT-3′; laminin B1 sense 5′-TGT CAG TCA CCT GCA GGA TG-3′ and antisense 5′-CAG GAT CCA GCA CAC GAT AG-3′; collagen IV α1 chain sense 5′-TCG GCT ATT CCT TCG TGA TG-3′ and antisense 5′-TCT CGC TTC TCT CTA TGG TG-3′; TGF-β1 sense 5′-ATA CAG GGC TTT CGC TTC AG-3′ and antisense

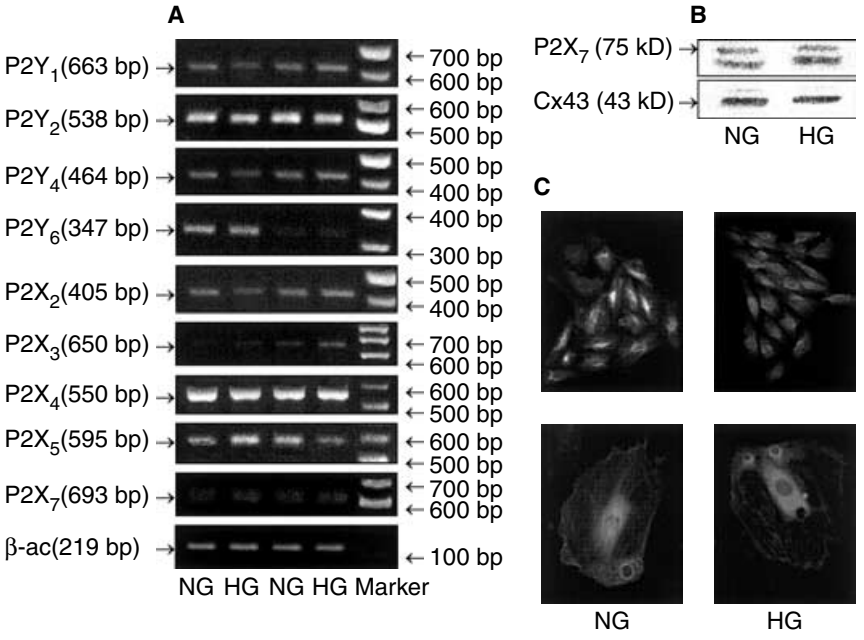

Fig. 1. P2YR and P2XR expression in rat mesangial cells grown under normal glucose (NG) and high glucose (HG) conditions for 10 to 15 days. (*A*) Gene expression of P2YRs, P2XRs and housekeeping β-actin (β-ac). Representative agarose gels of polymerase chain reaction products from two different cell lines and DNA markers. (*B*) Protein expression of $P2XR_7$ and reference protein connexin 43 (Cx43) (representative Western blot). (*C*) $P2XR_7$ protein expression, distribution, and pore formation (representative immunofluorescence images at low and high magnification, upper and bottom, respectively).

5′-GTC CAG GCT CCA AAT GTA GG-3′; and β-actin sense 5′-TCT AGG CAC CAA GGT GTG-3′ and antisense 5′-TCA TGA GGT AGT CCG TCA GG-3′. The mutants were made by creating a deletion in the original PCR product and preliminary experiments were performed to establish the range of mutant concentrations producing a slope of the line close to one and within which the equivalence point falls (approximately in the middle). After electrophoresis of PCR products, the ratio of unknown cDNA/mutant was quantified by scanning densitometry using the ImageJ software, a public domain Java image processing program inspired by NIH Image, and results were expressed as the ratio of each target to β-actin mRNA level.

Fibronectin content of monolayers extracted with 0.5 N NaOH and the amounts of fibronectin and TGF-β1 released in the medium during the 8 hours following incubation with purinergic agonists or antagonists were measured by enzyme-linked immunosorbent assay (ELISA), as previously reported [28]. The levels of fibronectin in cell extracts and conditioned media, were quantified using a rabbit polyclonal antibody against rat fibronectin (Calbiochem, San Diego, CA, USA), whereas release of TGF-β1 was measured using the Quantikine TGF-β1 Kit (R&D Systems), preceded by activation of TGF-β1 by acidification. To assess bioactive TGF-β1 levels, measurements were performed also without prior acidification using 20-fold concentrated media, obtained by lyophylization. Values were normalized to the DNA content of monolayers, as assessed fluorimetrically in 0.5 N NaOH extracts after reaction with 0.6 μmol/L 4,6-diamidino-2-phenylindole (Sigma Chemical Co.), as previously described [28].

Statistical analysis

Values were expressed as mean ± SD; the percent change was also calculated. Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for multiple comparisons. All statistical tests were performed on raw data.

RESULTS

Expression and functional activity of purinergic receptors

We first investigated P2R expression by RT-PCR. The following subtypes were found to be expressed: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, and $P2X_7$. Quantification of ethidium bromide bands at scanning densitometry did not show significant differences in P2Y or P2X expression in cells grown in high glucose vs. normal glucose and, therefore, we did not move on to competitive RT-PCR. Among P2XRs, $P2X_2$, $P2X_3$, and $P2X_7$ were weakly expressed, while $P2X_4$ and $P2X_5$ showed a strong band (Fig. 1A). As it has been previously reported that $P2X_7$ is present (though weakly expressed) and functional in rat mesangial cells [13, 18], we further assessed its expression by Western blot analysis. This confirmed $P2X_7$ expression, albeit at a rather low level and also showed a strong band of approximately 65 kD (Fig. 1B), as previously reported by us in fibroblasts [23] and other investigators in brain [29]. This band has been referred to as the nonglycosylated form of $P2X_7$ [29], though it was observed also in microglial cells from $P2X_7$ knockout mice [30].

Table 1. Adenosine triphosphate (ATP) levels and ecto-ATPase activity in rat mesangial cells grown under normal glucose vs. high glucose conditions (mean ± SD)

	Normal glucose	High glucose
ATP		
Extracellular *μmol/L*	0.52 ± 0.13 ($N = 6$)	1.08 ± 0.17 ($N = 6$)[a]
Intracellular *μmol/L*	1650 ± 440 ($N = 6$)	1460 ± 710 ($N = 6$)
EctoATPase activity *nmol inorganic phosphorus/ hour/3 × 10^5 cells*	29.4 ± 7.8 ($N = 7$)	31.0 ± 5.1 ($N = 7$)

[a]Significantly different vs. normal glucose ($P < 0.001$).

To confirm that P2Rs detected in rat mesangial cells were functionally active, we next assessed $[Ca^{2+}]_i$ in response to 1 mmol/L ATP. ATP stimulation produced an increase in $[Ca^{2+}]_i$ that did not differ between normal glucose and high glucose conditions (+115 ± 19 nmol/L vs. + 120 ± 28 nmol/L over baseline values). However, at variance with previous studies with human fibroblasts [31], basal $[Ca^{2+}]_i$ was slightly but significantly higher in rat mesangial cells cultured in high glucose vs. those in normal glucose (121 ± 10 nmol/L vs. 80 ± 16 nmol/L) ($P < 0.05$). Immunofluorescence analysis with anti-$P2X_7$ antibody showed ring-like membrane structures mainly localized at the periphery of the cell, clearly indicating pore formation, with no difference under high glucose vs. normal glucose conditions (Fig. 1C), thus ruling out the possibility that high glucose could have increased the recruitment of $P2X_7$ onto the plasma membrane.

Levels of eATP and ecto-ATPase activity

We previously reported that fibroblasts from type 2 diabetic patients release a higher amount of ATP into the pericellular space [31]. This suggested that either an inborn defect or the chronic exposure to hyperglycemia might be responsible for a higher spontaneous ATP secretion. Thus, we checked the effect of high glucose on the amount of ATP released by rat mesangial cells. Cells grown in high glucose secreted almost twice as much ATP as those in normal glucose, whereas total intracellular ATP levels were unchanged (Table 1). Iso-osmolar mannitol did not mimic the effect of high glucose on ATP secretion (0.49 ± 0.13 μmol/L), thus indicating that the increased ATP release under high glucose conditions was not dependent on enhanced cell death. This was supported also by the observation that both high glucose and iso-osmolar mannitol did not affect cell viability, as assessed by lactate dehyrogenase (LDH) release (15 ± 7 and 14 ± 7, respectively vs. 14 ± 5% in normal glucose) and tripan blue exclusion (not shown). Another possible explanation for the higher eATP level in high glucose might be a lower ecto-ATPase activity. However, measurement of inorganic phosphate accumulation under these conditions suggested that this was not the case (Table 1).

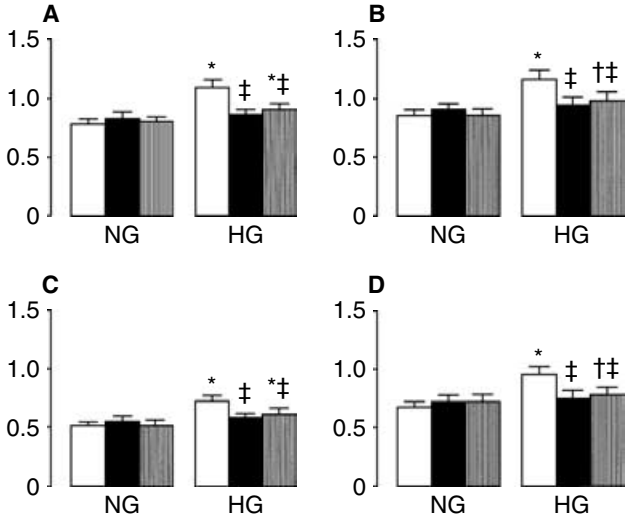

Fig. 2. mRNA levels of fibronectin (*A*), laminin B1 (*B*), collagen IV α1 chain (*C*), and transforming growth factor-β1 (*D*) (expressed as optical density ratio to β-actin mRNA level) in rat mesangial cells incubated with nucleotide scavengers. The cells were grown under normal glucose (NG) and high glucose (HG) conditions for 10 to 15 days, then left untreated (□) or incubated for 4 hours with hexokinase (50 μg/mL) (■) or apyrase (0.4 U/mL) (▨). Mean ± SD ($N = 6$ per experimental conditions). Significantly different vs. normal glucose at $^*P < 0.001$ or $\dagger P < 0.01$; and vs. high glucose at $\ddagger P < 0.001$.

ECM and TGF-β production

It is known that high glucose stimulates production of ECM proteins and TGF-β from rat mesangial cells [20]. Our study confirmed this fibrogenic effect of high glucose, that was not mimicked by iso-osmolar mannitol (not shown), thus ruling out a role for osmolarity, as previously reported [28].

In this work, we tested the hypothesis that eATP is involved in this response by assessing the effect of removal of eATP by addition of hexokinase or apyrase, two potent nucleotide scavengers. Treatment with these scavengers did not produce significant effects in rat mesangial cells grown in normal glucose, whereas it reduced substantially (by 60% to 70%) the gene expression of matrix proteins and TGF-β in rat mesangial cells grown in high glucose (Fig. 2), thus suggesting that the increased ATP release induced by high glucose may participate in the process of ECM accumulation.

The addition of increasing doses of exogenous ATP under normal glucose conditions produced a dose-dependent increase of fibronectin, laminin, collagen IV, and TGF-β1 mRNA levels, thus confirming that an excess of ATP stimulates matrix production. The same phenomenon was observed in high glucose, though the percent increase was lower than in normal glucose, due

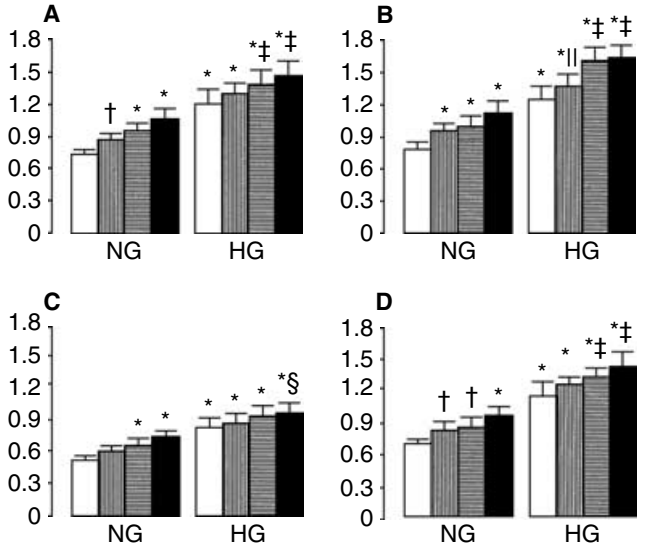

Fig. 3. mRNA levels of fibronectin (*A*), laminin B1 (*B*), collagen IV α1 chain (*C*), and transforming growth factor-β1 (*D*) (expressed as optical density ratio to β-actin mRNA level) in rat mesangial cells incubated with adenosine triphosphate. The cells were grown under normal glucose (NG) and high glucose (HG) conditions for 10 to 15 days, then left untreated (□) or incubated for 4 hours with increasing doses of adenosine triphosphate 0.05 mmol/L (▥), 0.10 mmol/L (▤), and 0.30 mmol/L (■). Mean ± SD ($N = 6$ per experimental conditions). Significantly different vs. normal glucose at $^*P < 0.001$ or $\dagger P < 0.01$; and vs. high glucose at $\ddagger P < 0.001$, $\S P < 0.01$, or $\| P < 0.05$.

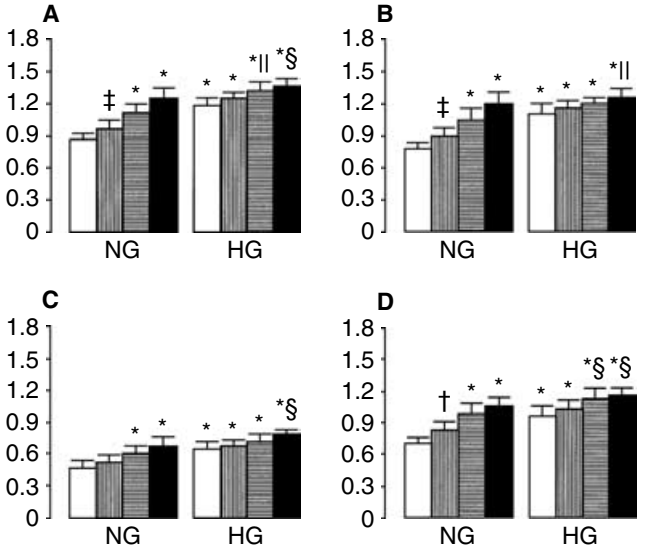

Fig. 4. mRNA levels of fibronectin (*A*), laminin B1 (*B*), collagen IV α1 chain (*C*), and transforming growth factor-β1 (*D*) (expressed as optical density ratio to β-actin mRNA level) in rat mesangial cells incubated with benzoyl-benzoyl adenosine triphosphate (B_z-ATP). The cells were grown under normal glucose (NG) and high glucose (HG) conditions for 10 to 15 days, then left untreated (□) or incubated for 4 hours with increasing doses of benzoyl-benzoyl adenosine triphosphate 0.05 mmol/L (▥), 0.10 mmol/L (▤), and 0.30 mmol/L (■). Mean ± SD ($N = 6$ per experimental conditions). Significantly different vs. normal glucose at $^*P < 0.001$, $\dagger P < 0.01$, or $\ddagger P < 0.05$; and vs. high glucose at $\S P < 0.001$ or $\| P < 0.01$.

to the marked stimulation of matrix production induced by high glucose per se (Fig. 3). The lower effective dose was 0.01 mmol/L (though increases were not significant for some of the targets examined) and no further increase was observed with doses >0.30 mmol/L (not shown).

Several ATP effects are mimicked by the analogue Bz-ATP, a strong and selective $P2X_4$ and $P2X_7$ agonist [32]. Figure 4 shows that this compound was a quite as potent agonist as ATP. As rat mesangial cells also express $P2Y_2$ and $P2Y_4$ that are strongly activated by UTP [33], we also tested the effect of UTP on matrix and TGF-β production. Figure 5 shows that, contrary to ATP and Bz-ATP, UTP caused a marked inhibition of fibronectin, laminin, collagen IV, and TGF-β1 mRNA expression, both under normal glucose and high glucose conditions. The potent $P2Y_1$ agonist 2-MeS-ATP [34] had no effect on matrix or TGF-β gene expression (not shown).

A potent, albeit not very selective, P2X antagonist is the Schiff base-forming compound oATP [35]. While oATP may also block non-P2Rs [36], it is useful in discriminating between P2XRs and P2YRs because P2YRs are not inhibited at oATP concentrations that completely wipe out any P2X activity [35]. Figure 6 shows that, under normal glucose, oATP had no effect on basal ECM and TGF-β gene expression, but reduced significantly (by 60%) that stimulated by ATP. Under high glucose, a condition that per se causes ECM and TGF-β up-regulation, oATP also reduced basal transcript level, though to a

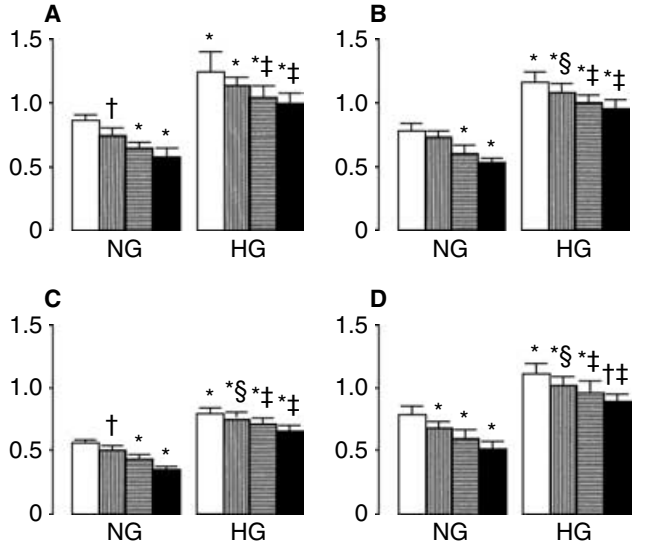

Fig. 5. mRNA levels of fibronectin (*A*), laminin B1 (*B*), collagen IV α1 chain (*C*), and transforming growth factor-β1 (*D*) (expressed as optical density ratio to β-actin mRNA level) in rat mesangial cells incubated with uridine triphosphate. The cells were grown under normal glucose (NG) and high glucose (HG) conditions for 10 to 15 days, then left untreated (□) or incubated for 4 hours with increasing doses of uridine triphosphate 0.05 mmol/L (▥), 0.10 mmol/L (▤) and 0.30 mmol/L (■). Mean ± SD ($N = 6$ per experimental conditions). Significantly different vs. normal glucose at $^*P < 0.001$ or $\dagger P < 0.01$; and vs. high glucose at $\ddagger P < 0.001$ or $\S P < 0.05$.

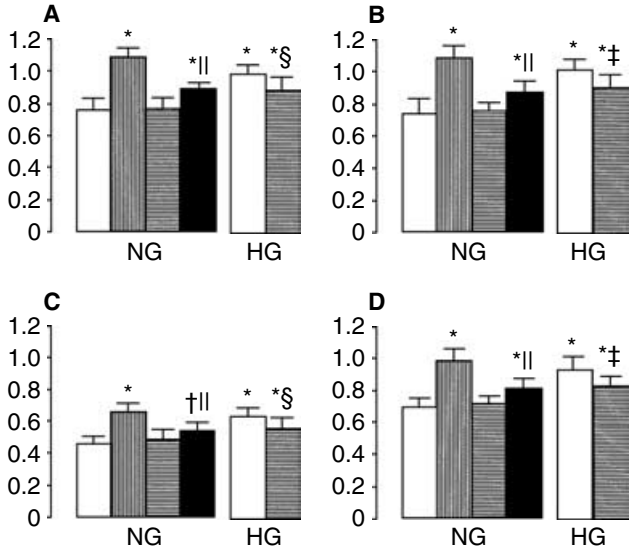

Fig. 6. mRNA levels of fibronectin (*A*), laminin B1 (*B*), collagen IV α1 chain (*C*), and transforming growth factor-β1 (*D*) (expressed as optical density ratio to β-actin mRNA level) in rat mesangial cells incubated with oxidized ATP. The cells were grown under normal glucose (NG) and high glucose (HG) conditions for 10 to 15 days, then left untreated (□) or incubated for 4 hours with 0.30 mmol/L adenosine triphosphate (ATP) (▥), for 2 hours with 0.30 mmol/L oxidized ATP (▤), or for 2 hours with 0.30 mmol/L oxidized ATP followed by 4-hour coincubation with 0.30 mmol/L ATP (■). Mean ± SD ($N = 6$ per experimental conditions). Significantly different vs. normal glucose at $^*P < 0.001$ or $\dagger P < 0.05$; vs. high glucose at $\ddagger P < 0.01$ or $\S P < 0.05$; and vs. ATP at $\| P < 0.001$.

lesser extent (by 40%). We also tested two additional inhibitors, the wide spectrum P2R antagonist PPADS [37] and the selective $P2Y_1$ blocker MRS-2179 [37], but neither of them affected significantly ECM or TGF-β gene expression (not shown).

The use of a TGF-β blocking antibody, but not nonimmune IgG, virtually prevented ATP-induced up-regulation of fibronectin, laminin, and collagen IV gene expression under normal glucose conditions (Fig. 7), thus suggesting a TGF-β–dependent mechanism for purinergic modulation of ECM production.

Cell and medium content of fibronectin (Table 2) and total TGF-β1 released from cells (Table 3) showed the same trend of mRNA expression. Bioactive TGF-β1 in conditioned media was also increased by high glucose (28.1 ± 2.9), 0.30 mmol/L ATP (24.7 ± 3.5) and Bz-ATP (25.3 ± 3.2) and reduced by 0.30 mmol/L UTP (12.9 ± 3.0) vs. normal glucose (17.1 ± 2.2 pg/μg DNA) ($P < 0.001$); moreover, hexokinase reduced significantly ($P < 0.001$) high glucose–induced up-regulation of bioactive TGF-β1 (22.7 ± 3.3).

DISCUSSION

The present paper reports on the effect of eATP on ECM and TGF-β production in rat mesangial cells grown

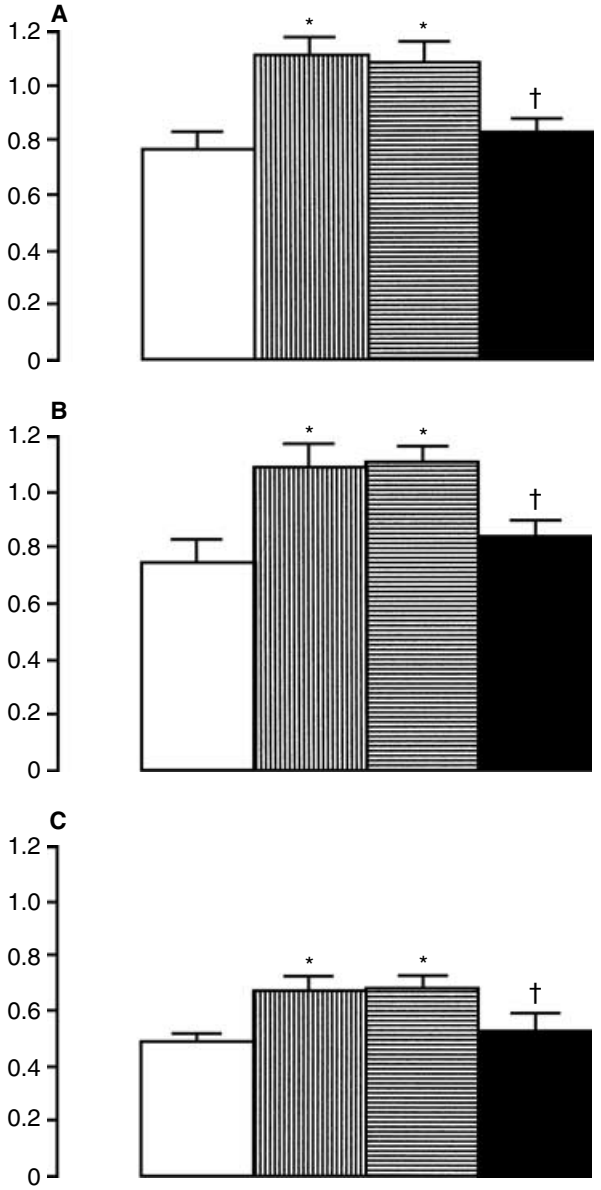

Fig. 7. mRNA levels of fibronectin (*A*), laminin B1 (*B*), and collagen IV α1 chain (*C*) (expressed as optical density ratio to β-actin mRNA level) in rat mesangial cells incubated with transforming growth factor β (TGF-β) neutralizing antibody. The cells were grown under normal glucose conditions for 10 to 15 days, then left untreated (□) or incubated for 4 hours with 0.30 mmol/L adenosine triphosphate (ATP) alone (▥), 0.30 mmol/L ATP plus 30 μg/mL nonimmune IgG (▤), or 0.30 mmol/L ATP plus 30 μg/mL TGF-β neutralizing antibody (■). Mean ± SD ($N = 6$ per experimental conditions). Significantly different vs. normal glucose at $^*P < 0.001$; and vs. ATP at $\dagger P < 0.001$.

under normal glucose and high glucose conditions. Taken together, our observations show that eATP might be involved in the pathogenesis of ECM accumulation and consequent mesangial expansion occurring in diabetic and other glomerular diseases.

The levels that eATP may reach in the extracellular space are a matter of debate and estimates range from

Table 2. Cell and medium fibronectin levels (μg/μg DNA) (mean ± SD) ($N = 6$ per experimental conditions) in rat mesangial cells grown under normal glucose vs. high glucose conditions ± various purinergic agonists or antagonists and nonimmune IgG or transforming growth factor-β (TGF-β) blocking antibody

	Cell		Medium	
	Normal glucose	High glucose	Normal glucose	High glucose
Untreated	2.081 ± 0.183	3.264 ± 0.322[a]	0.821 ± 0.074	1.344 ± 0.136[a]
+ hexokinase (50 μg/mL)	2.204 ± 0.357	2.749 ± 0.240[a,e]	0.867 ± 0.054	1.117 ± 0.142[a,d]
+ apyrase (4 U/mL)	2.159 ± 0.222	2.842 ± 0.397[a,e]	0.841 ± 0.091	1.167 ± 0.155[a,e]
Untreated	2.223 ± 0.161	3.183 ± 0.244[a]	0.954 ± 0.088	1.407 ± 0.155[a]
+ ATP (0.05 mmol/L)	2.422 ± 0.145	3.343 ± 0.136[a]	1.031 ± 0.066	1.501 ± 0.133[a]
+ ATP (0.10 mmol/L)	2.640 ± 0.155[a]	3.465 ± 0.154[a,e]	1.159 ± 0.078[b]	1.624 ± 0.154[a,d]
+ ATP (0.30 mmol/L)	2.964 ± 0.178[a]	3.664 ± 0.211[a,d]	1.277 ± 0.090[a]	1.728 ± 0.159[a,d]
Untreated	2.121 ± 0.123	3.082 ± 0.277[a]	0.911 ± 0.066	1.355 ± 0.143[a]
+ Bz-ATP (0.05 mmol/L)	2.324 ± 0.158	3.380 ± 0.367[a]	1.011 ± 0.076	1.467 ± 0.124[a]
+ Bz-ATP (0.10 mmol/L)	2.596 ± 0.178[a]	3.524 ± 0.344[a,e]	1.150 ± 0.070[a]	1.600 ± 0.144[a,d]
+ Bz-ATP (0.30 mmol/L)	2.786 ± 0.223[a]	3.764 ± 0.256[a,d]	1.249 ± 0.101[a]	1.689 ± 0.107[a,d]
Untreated	2.220 ± 0.118	3.101 ± 0.234[a]	0.976 ± 0.079	1.376 ± 0.124[a]
+ UTP (0.05 mmol/L)	2.021 ± 0.157	2.840 ± 0.289[a]	0.904 ± 0.080	1.294 ± 0.155[a]
+ UTP (0.10 mmol/L)	1.859 ± 0.178[b]	2.702 ± 0.345[a,d]	0.825 ± 0.056	1.229 ± 0.133[a]
+ UTP (0.30 mmol/L)	1.678 ± 0.218[a]	2.524 ± 0.265[a,d]	0.759 ± 0.082[a]	1.158 ± 0.124[a,d]
Untreated	2.111 ± 0.141	3.063 ± 0.261[a]	0.916 ± 0.059	1.287 ± 0.133[a]
+ ATP (0.30 mmol/L)	2.728 ± 0.201[a]	NA	1.211 ± 0.072[a]	NA
+ oATP (0.30 mol/L)	2.181 ± 0.217	2.745 ± 0.314[a,e]	0.967 ± 0.086	1.128 ± 0.078[a,e]
+ oATP + ATP (0.30 mmol/L)	2.433 ± 0.115[c,g]	NA	1.089 ± 0.056[a,g]	NA
Untreated	2.187 ± 0.154	NA	0.915 ± 0.067	NA
+ ATP (0.30 mmol/L)	3.067 ± 0.232[a]	NA	1.314 ± 0.087[a]	NA
+ ATP + IgG (30 μg/mL)	3.101 ± 0.172[a]	NA	1.297 ± 0.088[a]	NA
+ ATP + α-TGF-β (30 μg/mL)	2.381 ± 0.187[f]	NA	1.001 ± 0.043[f]	NA

Abbreviations are: ATP, adenosine triphosphate; Bz-ATP, benzoylbenzoylATP; oATP, oxidized ATP; α-TGF-β, TGF-β blocking antibody; NA, not assessed; UTP, uridine triphosphate.

Significantly different vs. normal glucose at [a] $P < 0.001$, [b] $P < 0.01$ or [c] $P < 0.05$; vs. high glucose at [d] $P < 0.001$ or [e] $P < 0.01$; and vs. ATP at [f] $P < 0.001$ or [g] $P < 0.05$.

the high micromolar to the nanomolar range [1], depending on the tissue/cell type examined and, particularly, the efficacy of ectoenzyme inactivation in the assay procedure. An accurate quantitation of eATP concentrations is further compounded by the likely occurrence of ATP secretion in protected intercellular compartments (e.g., at sites of close cell-to-cell interactions, see below) [39]. Some environmental conditions such as hypoxia/ischemia, stress injury, or membrane damage can induce a release of ATP in the extracellular space from several sources: platelets, endothelial cells, vascular smooth muscle cells, and inflammatory cells [1]. While it is already known that nondamaged cells may release ATP under experimental inflammatory conditions [40], this is the first report, to our knowledge, of an increased eATP release from cells under high glucose conditions (and the same phenomenon might occur at the level of other resident and nonresident glomerular cells). Several mechanisms are likely to mediate nonlytic ATP release, though none of them has been so far shown to be ubiquitous. These mechanisms include nonvesicular efflux pathways involving connexins or connexin-related proteins (pannexins), ATP-binding cassette proteins, stretch-activated channels, and the plasmalemmal voltage-dependent anion channel, and vesicular trafficking, due to stimulated or constitutive exocytosis, particularly in nonexcitable cells [41]. Accordingly, several intracellular second messenger systems have been shown to modulate ATP release, including elevation of $[Ca^{2+}]_i$ and activation of phosphoinositide 3-kinase and Rho kinase [42]. Thus, we can speculate that high glucose may promote ATP release by activating one or more of these signaling pathways, as previously shown in mesangial and other vascular cells [43–45]. In addition, oxidative stress, which is known to be induced by high glucose, might mediate glucose-stimulated ATP release, as suggested by the demonstration that hyperoxia enhances eATP levels by promoting the generation of reactive oxygen species in endothelial cells [46]. This enhanced ATP release occurred in the absence of changes in the expression and function of P2Rs, thus suggesting an activation of the purinergic system mainly driven by increased nucleotide level rather than by change of P2R number or activity. Activation of the purinergic loop seems to be mainly required for ECM and TGF-β production in high glucose, since incubation with ATP-hydrolyzing enzymes was without effect under normal glucose conditions. This is not surprising, since even the use of a blocking antibody against TGF-β1, the major cytokine involved in the up-regulation of ECM production in response to high glucose or hyperglycemia, has little effect on this parameter both in vitro under normal glucose [47] and in vivo in normoglycemic conditions [48]. Activation of rat mesangial cells responses by exogenous ATP required doses in the medium-high micromolar range, similar to the threshold for purinergic receptor activation noted in other studies using mesangial

Table 3. Medium transforming growth factor-β (TGF-β1) levels (ng/μg DNA) (mean ± SD) ($N = 6$ per experimental conditions) in rat mesangial cells grown under normal glucose vs. high glucose conditions ± various purinergic agonists or antagonists

	Normal glucose	High glucose
Untreated	0.74 ± 0.09	1.05 ± 0.12[a]
+ hexokinase (50 μg/mL)	0.72 ± 0.07	0.87 ± 0.12[e]
+ apyrase (4 U/mL)	0.78 ± 0.06	0.90 ± 0.14[c,e]
Untreated	0.71 ± 0.08	1.02 ± 0.09[a]
+ ATP (0.05 mmol/L)	0.79 ± 0.04	1.09 ± 0.12[a]
+ ATP (0.10 mmol/L)	0.82 ± 0.05	1.15 ± 0.08[a,e]
+ ATP (0.30 mmol/L)	0.91 ± 0.07[a]	1.23 ± 0.10[a,d]
Untreated	0.76 ± 0.07	1.04 ± 0.10[a]
+ Bz-ATP (0.05 mmol/L)	0.86 ± 0.09	1.10 ± 0.08[a]
+ Bz-ATP (0.10 mmol/L)	0.90 ± 0.06[c]	1.16 ± 0.12[a,e]
+ Bz-ATP (0.30 mmol/L)	0.99 ± 0.08[a]	1.27 ± 0.14[a,d]
Untreated	0.72 ± 0.07	1.03 ± 0.12[a]
+ UTP 0.05 (mmol/L)	0.65 ± 0.06	0.97 ± 0.11[a]
+ UTP (0.10 mmol/L)	0.59 ± 0.08	0.90 ± 0.10[a]
+ UTP (0.30 mmol/L)	0.54 ± 0.07[b]	0.86 ± 0.14[b,e]
Untreated	0.75 ± 0.06[a]	1.03 ± 0.13[a]
+ ATP (0.30 mmol/L)	0.96 ± 0.08	NA
+ oATP (0.30 mmol/L)	0.74 ± 0.07	0.90 ± 0.07[a,e]
+ oATP + ATP (0.30 mmol/L)	0.84 ± 0.04[f]	NA

Abbreviations are: ATP, adenosine triphosphate; Bz-ATP, benzoylbenzoylATP; oATP, oxidized ATP; UTP, uridine triphosphate; NA, not assessed.

Significantly different vs. normal glucose at [a] $P < 0.001$, [b] $P < 0.01$ or [c] $P < 0.05$; vs. high glucose at [d] $P < 0.001$ or [e] $P < 0.05$; and vs. ATP at [f] $P < 0.05$.

and other glomerular cells [9, 13, 15–18] and also different cell types [37]. Conversely, the bulk concentrations measured by the luciferase-D-luciferin assay in rat mesangial cells grown under high glucose conditions, though almost doubled than those in normal glucose, were still in the low micromolar range, in keeping with previous reports in other stimulated cell systems [1]. However, as discussed by many other investigators and elegantly shown in recent studies in activated platelets [39], local ATP concentrations near the cell surface, and, accordingly, near the purinergic receptors might be much higher than those measured in the conditioned media also in mesangial cells, thus explaining this apparent discrepancy.

ATP is a wide range P2R agonist, since it activates several P2Rs of both subtypes. As previously reported in rat mesangial cells for cell proliferation and death [13–18], P2YRs and P2XRs may exert opposite actions, the net effect of ATP being the result of these actions. Thus, in order to identify the specific role of each P2R subtype and the individual receptor(s) involved in the modulation of ECM production, we performed a molecular and pharmacologic characterization.

The analysis of P2R expression confirmed and extended previous observations showing that RMC express the several P2XRs and P2YRs [13, 14]. The $P2X_7$ agonist Bz-ATP mimicked the effect of ATP, whereas the $P2X_7$ irreversible inhibitor oATP partially prevented it, thus suggesting a prosclerotic role for this receptor. The observation that attenuation of the effect of high glucose by oATP was less evident than the reduction of ATP-induced changes might be related to the multiple mechanisms involved in the prosclerotic effect of hyperglycemia, part of which are not dependent on the activation of the purinergic system. Biochemical analysis confirmed the expression of $P2X_7$ at both the mRNA and protein level, even if at low transcript level despite its relevant functional activity, as previously reported by others [13]. In addition to $P2X_7$, whose functional role has been partly elucidated [37], other P2XRs were also detected in rat mesangial cells (i.e., the subtypes $P2X_2$, $P2X_3$, $P2X_4$ and $P2X_5$). Though their function is still unclear, one of them, $P2X_4$, shares some properties with $P2X_7$, in particular the ability to form nonselective plasma membrane pores [49].

At variance with Bz-ATP, UTP, an uracil nucleotide, which is the preferred agonist at $P2Y_2$ and $P2Y_4$ and an effective stimulus for $P2Y_6$ [33], strongly reduced ECM and TGF-β mRNA and protein expression in a dose-dependent fashion, thus indicating that P2YRs exert an opposite effect on matrix production.

The prevention of ECM up-regulation by the use of a TGF-β blocking antibody indicates that purinergic modulation of ECM production is TGF-β–dependent and suggests that, under high glucose conditions, glucose and ATP may act in a synergistic or additive manner to stimulate TGF-β production. This is supported by the demonstration that both high glucose [50] and eATP [16] are capable of activating the MAPK cascade.

Desensitization of P2YRs has been demonstrated in different cell lines and preparations [51]. Both under high glucose conditions and upon addition of exogenous ATP, the pro-sclerotic activity of P2XRs seems to prevail over that of P2YRs, which might be in a state of chronic desensitization, as already shown in other cell types under the same experimental conditions [52]. However, this is in contrast with the report that similar doses of ATP produced a stimulation of rat mesangial cell proliferation that was mimicked by equimolar UTP, whereas Bz-ATP produced the opposite effect, thus indicating that, under those circumstances, exogenous ATP acted primarily via P2YRs [13]. The fact that 2-MeS-ATP, a specific $P2Y_1$ agonist, did not produce any significant effect might be explained by the minor role played by $P2Y_1$, as further confirmed by the lack of effect of the selective $P2Y_1$ antagonist MRS-2179.

Taken together, our results, together with previously reported data [13–18], have several important implications in the pathogenesis of diabetic and other glomerular diseases. In fact, both cell and matrix turnover were shown to be modulated by the purinergic system at the glomerular/mesangial level. Thus, the main, strictly related processes that underlie both maintenance of normal tissue homeostasis and development of glomerular diseases [53] would be influenced by eATP levels, which in turn might be affected by either chronic hyperglycemia or acute inflammation with increased ATP release from

platelets and other cells (and possibly up-regulation of $P2XR_7$ [14]). The resulting effect would be dependent on the prevalence of the effect of P2XRs, with consequent apoptosis and matrix deposition, or P2YRs, leading to proliferation and inhibition of ECM accumulation. Mechanisms regulating this balance are as yet unclear and deserve further investigation. In particular, elucidation of P2YR desensitization and resensitization would be extremely important for the understanding of the physiologic role of extracellular nucleotides and their possible therapeutic use in glomerular diseases.

Among the various biochemical mechanisms implicated in the injurious effect of hyperglycemia, PKC activation seems to play a major role [54]. Recent evidence suggests a link between PKC and the purinergic system. In fact, PKC activation was shown to induce desensitization of $P2Y_2$ in rat mesangial cells [55], that is in keeping with our finding of a down-regulatory effect of P2YRs on mesangial matrix production. Moreover, stimulation of rat mesangial cells with ATP and UTP leads to a rapid activation of the PKB pathway, a cascade known to play an important role in the antiapoptotic, but not inflammatory or mitogenic response [56].

CONCLUSION

The purinergic system appears to be involved in the modulation of ECM production in rat mesangial cells, with eATP exerting a dual effect: a stimulatory, probably via $P2X_7$, and an inhibitory, via a P2YR. In the presence of elevated eATP levels, due to increased release from mesangial (and other glomerular or nonresident) cells, as in response to high glucose concentrations (and possibly ischemic or inflammatory conditions), ECM proteins accumulate as a result of a functional predominance of P2XRs (electively stimulated by higher ATP concentrations) over P2YRs. This mechanism might participate in the pathogenesis of mesangial expansion occurring in diabetic and possibly any other glomerular disease associated with increased ATP release from resident or nonresident glomerular cells.

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